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Special Lecture

The Pezcoller Lecture: Cancer Cell Cycles Revisited

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Abstract

Genetic lesions that disable key regulators of G_1 phase progression in mammalian cells are present in most human cancers. Mitogen-dependent, cyclin D-dependent kinases (cdk4 and cdk6) phosphorylate the retinoblastoma (Rb) tumor suppressor protein, helping to cancel its growth-inhibitory effects and enabling E2F transcription factors to activate genes required for entry into the DNA synthetic phase (S) of the cell division cycle. Among the E2F-responsive genes are cyclins E and A, which combine with and activate cdk2 to facilitate S phase entry and progression. Accumulation of cyclin D-dependent kinases during G_1 phase sequesters cdk2 inhibitors of the Cip/Kip family, complementing the effects of the E2F transcriptional program by facilitating cyclin E-cdk2 activation at the G_1-S transition. Disruption of "the Rb pathway" results from direct mutational inactivation of Rb function, by overexpression of cyclin D-dependent kinases, or through loss of $p16^{INK4a}$, an inhibitor of the cyclin D-dependent kinases. Reduction in levels of $p27^{Kip1}$ and increased expression of cyclin E also occur and carry a poor prognostic significance in many common forms of cancer. The ARF tumor suppressor, encoded by an alternative reading frame of the *INK4a-ARF* locus, senses "mitogenic current" flowing through the Rb pathway and is induced by abnormal growth promoting signals. By antagonizing Mdm2, a negative regulator of the p53 tumor suppressor, ARF triggers a p53-dependent transcriptional response that diverts incipient cancer cells to undergo growth arrest or apoptosis. Although ARF is not directly activated by signals that damage DNA, its loss not only dampens the p53 response to abnormal mitogenic signals but also renders tumor cells resistant to treatment by cytotoxic drugs and irradiation. Lesions in the p16 — cyclin D-CDK4 — Rb and ARF — Mdm2 — p53 pathways occur so frequently in cancer, regardless of patient age or tumor type, that they appear to be part of the life history of most, if not all, cancer cells.

Introduction

"The greatest single achievement of nature to date was surely the invention of the molecule of DNA. We have had it from the very beginning, built into the first cell to emerge, membranes and all, somewhere in the soupy water of the cooling planet three thousand million years or so ago. All of today's DNA, strung through all of the cells of the earth, is simply an extension and elaboration of that first molecule. In a fundamental sense, we cannot claim to have made progress, since the method used for growth and replication is essentially unchanged" (1).

As the late Lewis Thomas implied, the principle task of the cell division cycle is to replicate DNA (without errors during S phase) and to segregate the duplicated chromosomal DNA equally to two daughter cells [during mitosis (or M phase)] (Fig. 1). In addition to the molecular regulators that drive these processes, a monitoring circuitry ensures that S phase is completed before mitosis begins and *vice versa*. Early embryonic cell cycles exhibit rapidly alternating S and M phases without gap phases between them. This suggests that the gap phases seen in somatic cell cycles–G_1 separating the M and S phases, and G_2 separating the S and M phases–are not strictly essential for the correct operation of the cell cycle engine. It is intriguing to reflect on this point, because many of the G_1 phase regulators that prove so important in accelerating or braking the cell cycle engine of mammalian cells are encoded by nonessential genes, whose elimination from the germ line needs not lead to deleterious effects on organismal development.

G_1 phase is the interval in which cells respond to extracellular cues that ultimately determine whether cells will make the decision to replicate DNA and divide or, alternatively, to exit the cell cycle into a quiescent state (G_0). Once cells make the decision to begin DNA replication, they are irreversibly committed to complete the cycle, and the time late in G_1 phase at which this decision is made was designated the "restriction point" by Arthur Pardee (reviewed in Ref. 2; Fig. 1). When cells are stimulated by growth factors to enter the cycle from G_0, they generally require continuous mitogenic stimulation to be driven to the restriction point, after which mitogens can be withdrawn and cells will enter S phase and complete the cycle in their absence. Conversely, antiproliferative compounds, such as cytokines like transforming growth factor-β or drugs such as rapamycin, can only arrest the proliferation of cells that are progressing through G_1 phase but have not yet reached the restriction point. In mammalian cells, progression through the restriction point involves a series of events that lead to, but are distinct from, the firing of replication origins and that temporally precede the G_1-S transition by several hours (Fig. 1).

G1 Cyclins and cdks

In general, cell cycle transitions are controlled by cdks.[2] These holoenzymes contain both regulatory (cyclin) and catalytic (cdk) subunits but likely exist as higher order complexes that include additional proteins (see below). Restriction point control is mediated by two families of enzymes, the cyclin D- and E-dependent kinases. The D-type cyclins (D1, D2, and D3; Refs. 3–5) interact combinatorially with two distinct catalytic partners (cdk4 and cdk6; Refs. 6 and 7) to yield at least six possible holoenzymes that are expressed in tissue-specific patterns. Whereas cdk4 and cdk6 are relatively long-lived proteins, the D-type cyclins are unstable, and their induction, synthesis, and assembly with their catalytic partners all depend upon persistent mitogenic signaling. In this sense, the D-type cyclins act as growth factor sensors, forming active kinases in response to extracellular cues (reviewed in Ref. 8).

The mitogen-dependent accumulation of the cyclin D-dependent kinases triggers the phosphorylation of Rb, thereby helping to cancel its growth-repressive functions (6, 9, 10). Rb represses the transcription of genes whose products are required for DNA synthesis. It does so by binding transcription factors such as the E2Fs (reviewed in Ref. 11) and recruiting repressors such as histone deacetylases (12–14) and chromosomal remodeling SWI/SNF complexes (15) to E2F-responsive promoters on DNA. However, Rb phosphorylation by the G_1

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[2] The abbreviations used are: cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; ARF, alternative reading frame; MEF, mouse embryo fibroblast.

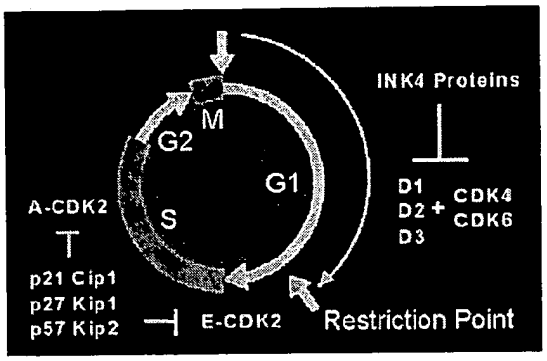

Fig. 1. G1 cdks and cdk inhibitors. Somatic cell cycles consist of alternating DNA synthetic (*S*) and mitotic (*M*) phases, separated by gap phases (*G1* and *G2*) as indicated. Mammalian cells respond to extracellular mitogens and antiproliferative cytokines from the time that they exit mitosis (*vertical arrow* at *top*) until they reach the restriction point, after which they can complete the cell division cycle in the absence of extracellular growth factors. Cyclin D-dependent kinases accumulate in response to mitogenic signals and initiate the phosphorylation of Rb, a process that is completed by cyclin E-cdk2. Once cells enter S phase, cyclin E is degraded and cyclin A enters into complexes with cdk2. INK4 proteins oppose the activities of the various cyclin D-dependent kinases, whereas Cip/Kip proteins specifically inhibit cyclin E-cdk2 and cyclin A-cdk2.

cdks disrupts these interactions (15, 16), enabling untethered E2Fs to function as transcriptional activators (Fig. 2). Apart from a battery of genes that regulate DNA metabolism, E2Fs induce the cyclin E and A genes. Cyclin E enters into a complex with its catalytic partner cdk2 (17–20) and collaborates with the cyclin D-dependent kinases to complete Rb phosphorylation (Fig. 2; Refs. 21–26). This shift in Rb phosphorylation from mitogen-dependent cyclin D-cdk4/6 complexes to mitogen-independent cyclin E-cdk2 accounts in part for the loss of dependency on extracellular growth factors at the restriction point. Cyclin E-cdk2 also phosphorylates substrates other than Rb, and its activity is somehow linked to replication origin firing (27, 28). The activity of the cyclin E-cdk2 complex peaks at the G_1-S transition, after which cyclin E is degraded and replaced by cyclin A (Fig. 1).

Cdk Inhibitors: The Cip/Kip Family

The actions of cdk2 are opposed by the *Cip/Kip* family of polypeptide inhibitors that includes $p21^{Cip1}$ (29–31), $p27^{Kip1}$ (32–34), and $p57^{Kip2}$ (Refs. 35 and 36; reviewed in Ref. 37; Fig. 1). In quiescent cells, the levels of $p27^{Kip1}$ are generally high. However, as cells enter cycle and accumulate cyclin D-dependent kinases, the Cip/Kip proteins are sequestered in complexes with cyclin D-dependent cdks (Fig. 2). Although it was initially assumed that the Cip/Kip proteins would inhibit both cdk4/6 and cdk2, we now recognize that the Cip/Kip-bound cyclin D-dependent enzymes remain catalytically active (38–41). Even more surprisingly, it turns out that Cip/Kip proteins are required for the assembly of the active cyclin D-dependent holoenzymes (Refs. 40 and 41; Fig. 2). In cycling cells, virtually all of the $p27^{Kip1}$ molecules remain associated with cyclin D-cdk complexes. However, mouse embryo fibroblasts taken from animals lacking both the *Kip1* and *Cip1* genes–$p57^{Kip2}$ is not synthesized in these cells–express no detectable cyclin D-dependent kinase activity and still have relatively unperturbed cell cycle transit times (41). In this setting, the levels of cyclin E-cdk2 activity are greatly increased and are apparently sufficient to phosphorylate Rb. Together, these data point to a second noncatalytic role of the cyclin D-dependent kinases, *i.e.*, the mitogen-dependent accumulation of cyclin D-dependent kinases sequesters Cip/Kip proteins, thereby facilitating cyclin E-cdk2 activation (reviewed in Ref. 42). This complements the Rb-E2F transcriptional program (see above) and helps make the appearance of cyclin E-cdk2 activity contingent upon accumulation of cyclin D-cdk4/6-Cip/Kip complexes (Fig. 2).

Once cyclin E-cdk2 is activated, it phosphorylates $p27^{Kip1}$. This converts $p27^{Kip1}$ to a form that is recognized by ubiquitin ligases and is targeted for destruction in proteasomes (43–47). Therefore, cyclin E-cdk2 antagonizes the action of its own inhibitor (Fig. 2). It follows that once cyclin E-cdk2 is activated, unbound $p27^{Kip1}$ is rapidly degraded, contributing to the irreversibility of passage through the restriction point. If cells are persistently stimulated with mitogens, cyclin D-dependent kinase activity remains high in subsequent cycles, $p27^{Kip1}$ levels stay low, and virtually all of the $p27^{Kip1}$ can be found in complexes with the cyclin D-cdks. However, when mitogens are withdrawn, cyclin D is rapidly degraded, and previously sequestered Cip/Kip proteins are mobilized to inhibit cyclin E-cdk2, thereby arresting progression usually within a single cycle.

The *Kip1* gene was not initially thought to be a tumor suppressor, because both copies of the gene were not found to be deleted or silenced in tumor cells. Yet, there is now compelling evidence that *Kip1* is haplo-insufficient for tumor suppression, with loss of only one copy of the gene being sufficient to contribute to cancer (48). In retrospect, we might rationalize this finding through the realization that Cip/Kip proteins are essential for the formation of cyclin D-dependent holoenzymes, although, at least experimentally, $p21^{Cip1}$ can functionally replace $p27^{Kip1}$ in this regard. Still, low levels of $p27^{Kip1}$ (which can be associated with monoallelic *Kip1* deletions in tumor cells) combined with high levels of cyclin E are generally indicative of reduced long-term survival in various forms of cancer. This has been well documented in breast cancer, where the levels of $p27^{Kip1}$ and cyclin E in primary tumors have greater prognostic power than other markers (49, 50). It is particularly important in women without apparent lymph node involvement, in whom the choice of therapy critically depends on strongly predictive markers of this type.

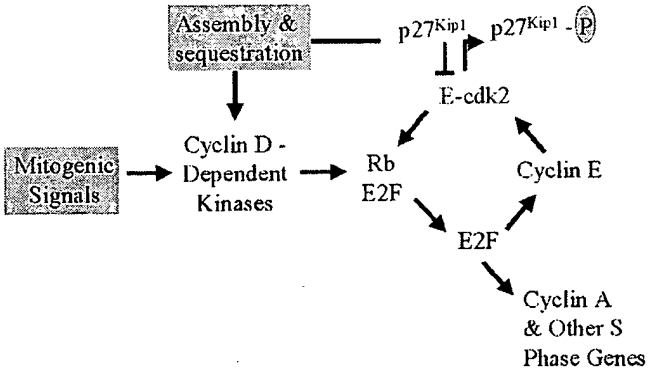

Fig. 2. Restriction point control and the G_1-S transition. As cells enter the division cycle from quiescence, the assembly of cyclin D-dependent kinases in response to mitogenic signals requires Cip/Kip proteins, which are incorporated into catalytically active holoenzyme complexes. The cyclin D-dependent kinases initiate Rb phosphorylation, releasing E2F from negative constraints and facilitating activation of a series of E2F-responsive genes, the products of which are necessary for S-phase entry. Activation of cyclin E by E2F enables formation of the cyclin E-cdk2 complex. This is accelerated by the continued sequestration of Cip/Kip proteins into complexes with assembling cyclin D-cdk complexes. Cyclin E-cdk2 completes the phosphorylation of Rb, further enabling activation of E2F-responsive genes, including cyclin A. Cyclin E-cdk2 also phosphorylates $p27^{Kip1}$, targeting it for ubiquitination and proteasomal degradation. The initiation of the self-reinforcing E2F transcriptional program together with degradation of $p27^{Kip1}$ alleviates mitogen dependency at the restriction point and correlates with the commitment of cells to enter S phase. In subsequent cycles, cyclin D-dependent kinases remain active as long as mitogens are present, and levels of $p27^{Kip1}$ remain low. All $p27^{Kip1}$ in cycling cells is complexed with cyclin D-cdk complexes. Mitogen withdrawal results in cyclin D degradation, liberating $p27^{Kip1}$ from this latent pool. The resulting inhibition of cyclin D- and E-dependent kinases leads to cell cycle arrest, usually within a single cycle.

Cdk Inhibitors: The INK4 Family

Another class of cdk inhibitors, the so-called INK4 proteins (named for their ability to inhibit cdk4), specifically target the cyclin D-dependent kinases (reviewed in Refs. 37 and 51; Fig. 1). INK4 proteins sequester cdk4/6 into binary cdk-INK4 complexes, liberating bound Cip/Kip proteins, and thereby indirectly inhibiting cyclin E-cdk2 to ensure cell cycle arrest (reviewed in Ref. 42). The ability of INK4 proteins to arrest the cell cycle in G_1 phase depends upon the presence of a functional Rb protein, implying that by inhibiting cyclin D-dependent kinases, Rb remains hypophosphorylated and able to repress transcription of S-phase genes (52–54). Note that disruption of cyclin D-cdk complexes and release of bound Cip/Kip proteins is insufficient to inhibit cyclin E-cdk2 in Rb-negative cells. This is likely attributable to the fact that cyclin E-cdk2 activity is normally under Rb-E2F control (Fig. 2), so that cells lacking Rb exhibit greatly elevated cyclin E-cdk2 kinase activity. This enables a conceptually simplified view of the "Rb pathway": INK4 proteins — cyclin D-dependent kinases — Rb — E2Fs → S phase entry.

To date, four INK4 proteins have been identified. These include the founding member $p16^{INK4a}$ (55) and three other closely related genes designated $p15^{INK4b}$ (56), $p18^{INK4c}$ (52, 57), and $p19^{INK4d}$ (57, 58). In humans, *INK4a* and *INK4b* are closely linked on the short arm of chromosome 9 (59), whereas *INK4c* maps to chromosome 1 and *INK4d* maps to chromosome 19. In mice, the *INK4c* and *INK4d* genes are expressed in stereotypic patterns in different tissues during development *in utero*, whereas *INK4a* and *INK4b* expression has not been detected prenatally (60). Gene disruption experiments in mice have revealed no overt effects of *INK4b* or *INK4d* loss (61). In contrast, mice lacking *INK4c* are similar to those lacking *Kip1* in the sense that they have organomegaly and pituitary tumors (62). "Pure" *INK4a*-null mice have not yet been produced (see below). However, one report has provided evidence that inbred BALB/c mice contain defective *INK4a* alleles that encode $p16^{INK4a}$ proteins incapable of inhibiting cyclin D-dependent kinases (63). At face value, the collective data argue that disabling single INK4 family members does not particularly increase the rate of spontaneous tumor development in mice.

Nonetheless, there is compelling evidence that *INK4a* loss-of-function occurs frequently in human cancers (reviewed in Ref. 51). In some familial melanomas, for example, one defective copy of *INK4a* is inherited, whereas the second is lost in tumor cells, the reduction to homozygosity being a classic feature of a tumor suppressor gene (59). In many forms of sporadic cancer, *INK4a* function is also lost (51). For example, virtually all pancreatic carcinomas exhibit *INK4a* defects. As might be expected, the loss of *INK4a* represents only one of several ways in which the Rb pathway can be disabled. In glioblastomas, CDK4 is frequently amplified, and *INK4a* function is lost in other cases. In small cell lung cancer, ~85% of tumors sustain Rb loss, whereas the remaining tumors exhibit *INK4a* loss-of function (10%) or cyclin D amplification (5%; reviewed in Refs. 51 and 64). A remaining puzzle is why other members of the INK4 gene family are not similarly targeted in human tumors. It therefore seems that *INK4a* plays a special role in tumor surveillance in humans. Whatever the reason for the preferential involvement of $p16^{INK4a}$, the available data have led to the reasonable speculation that disruption of the Rb pathway is part of the life history of many, if not all, human tumor cells (reviewed in Ref. 64).

The ARF Tumor Suppressor

Surprisingly, the *INK4a* gene encodes a second potent tumor suppressor (65, 66). The sequences encoding $p16^{INK4a}$ are embodied in three exons (designated 1α, 2, and 3), which specify an mRNA transcript of ~1 kb. In the human and mouse genomes, an alternative first exon (designated 1β) lies 15–20 kb upstream of the $p16^{INK4a}$ coding sequences, and its RNA is spliced to the exon 2 and exon 3 RNA segments to yield a second ~1 kb "β mRNA" whose 5′ end differs from the α transcript (65, 67–69). Alternative promoters located 5′ of exons 1α and 1β govern the independent production of the two mRNAs. The unusual feature is that the initiation codons within exons 1α and 1β are in different reading frames and, when spliced to the same sequences in exon 2, encode two distinct proteins that bear no relationship to one another (65). In the mouse, the ARF protein is represented by 64 amino acids encoded by exon 1β and 105 amino acids specified by exon 2. Mouse $p19^{ARF}$ is a highly basic protein that, when overexpressed, can cause cell cycle arrest in both the G_1 and G_2 phases of the cell cycle (65). Its human counterpart ($p14^{ARF}$) contains fewer exon 2-coded amino acids and is of lower molecular mass, but it has the same ability to induce cell cycle arrest.

Mice containing disrupted *INK4a/ARF* exon 2 sequences (70) or lacking only the *ARF* exon 1β sequences (66) are highly tumor prone and die of cancers within 15 months of age. The most predominant tumors are sarcomas, followed by lymphomas, carcinomas, and tumors of the central nervous system (71). *ARF*+/− heterozygotes develop tumors after a considerably longer latency, and the tumor cells lose the wild-type *ARF* allele, as is characteristic of a classical tumor suppressor gene. When MEFs of *INK4a/ARF* or *ARF*-null animals are explanted into culture and passaged on a defined 3T3 protocol, the cells do not senesce but rather continue to proliferate as established cell lines (66, 70). Normally, primary MEFs are generally resistant to transformation by oncogenic *Ras* and require the introduction of a so-called immortalizing oncogene, such as adenovirus *E1A* or *Myc*, to undergo transformation (72, 73). However, like established rodent fibroblast lines, *ARF*-null cells can be transformed by oncogenic *Ras* alone (66, 70). In these respects, *ARF*-null MEFs are similar to *p53*-deficient mouse fibroblasts, which are also immortal and can be transformed by Ras without a requirement for Myc or E1A. Moreover, spontaneously immortalized cells derived from a 3T3 protocol contain either mutations in the *p53* gene (80%) or exhibit bi-allelic *ARF* loss (the remaining 20%; Refs. 66 and 74). Together, these data suggested that ARF and p53 functioned in the same biochemical pathway.

p53 is a homotetrameric transcription factor that induces either cell cycle arrest or apoptosis, depending on the biological setting (reviewed in Refs. 75 and 76). Introduction of *ARF* into cells results in p53-dependent cell cycle arrest, indicating that ARF acts "upstream" of p53 (66). Cells lacking p53 alone are refractory to ARF-induced arrest, and in this setting, ARF protein expression is greatly increased. This suggests that p53 suppresses *ARF* expression through negative feedback, and consistent with this interpretation, reintroduction of p53 into these cells returns ARF protein expression to lower levels (Refs. 77 and 78; Fig. 3). ARF stabilizes p53 by antagonizing the p53-negative regulator Mdm2 (77–80). Mdm2 binds to the transactivation domain of the p53 tetramer to inhibit p53-dependent gene expression (81, 82), and it also manifests a ubiquitin ligase activity that appears to target p53 for proteasomal degradation (83). Intriguingly, Mdm2 is itself a p53-responsive gene that normally acts in feedback control to terminate the p53 response (Refs. 84 and 85; Fig. 3). ARF can interfere with all of the known functions of Mdm2, including its ability to: (*a*) block p53 transcription (77–79); (*b*) to ubiquinate p53 (86); and (*c*) to enforce p53 transport into the cytoplasm (87–89), where it is degraded in proteasomes (90–92).

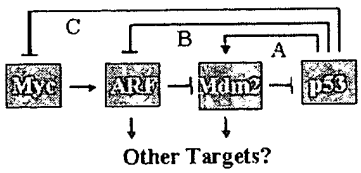

Fig. 3. The ARF-Mdm2-p53 pathway. In response to proteins such as Myc and E2F1 (not shown), ARF protein accumulates and antagonizes the activity of Mdm2. The resulting accumulation of p53 leads to cell cycle arrest or apoptosis, depending on the biological setting. Mdm2 is a p53-responsive gene (pathway *A*) whose p53-dependent accumulation helps to terminate the p53 response. In addition, p53 negatively regulates both ARF (pathway *B*) and Myc (pathway *C*) through as yet unknown mechanisms. ARF likely interacts with targets other than Mdm2, whereas Mdm2 may also functionally interact with proteins other than p53. [This figure is adapted from Eischen *et al.* (100) and is reprinted with permission from *Genes & Development.*]

ARF Connects Rb and p53

ARF expression is activated by abnormal mitogenic signals induced by overexpression of oncoproteins such as Myc (93), E1A (94), E2F1 (95), Ras (96), and v-Abl (97). In this manner, ARF serves to connect the Rb pathway with Mdm2 and p53 (Fig. 4). ARF acts as a fuse to "gate" inappropriate mitogenic signals flowing through the cyclin D-cdk –Rb –E2F circuit, inducing p53 under conditions in which abnormal proliferative signals are generated. This mode of cell-autonomous tumor surveillance diverts cells that have received an oncogenic insult to undergo p53-dependent growth arrest or apoptosis, thereby preventing incipient cancer cells from emerging as overt tumors (reviewed in Ref. 98).

But, if genes like Myc and E1A can induce *ARF* and p53 to trigger growth arrest or cell death, how can these same genes immortalize normal cells and collaborate with oncogenic Ras to transform them? A reasonable hypothesis is that Myc and E1A overexpression, by inducing both cell proliferation and compensating p53-dependent apoptosis, selects for resistant cells that have sustained mutations in the ARF-Mdm2-p53 pathway and that can now be transformed by oncogenic *Ras* alone. To test this idea, primary MEFs were infected with a high titer *Myc* retrovirus, and Myc-induced apoptosis was enforced by depriving the infected cells of serum-containing survival factors. Rare surviving cells were then recloned, expanded as colonies, and genotyped for *p53* mutations and/or *ARF* loss. Strikingly, all such colonies lost the function of p53 or ARF but not both (93). In short, ARF normally acts to protect cells from Myc overexpression by facilitating Myc-induced, p53-dependent apoptosis. Cells corrupted in the ARF-Mdm2-p53 pathway are resistant to Myc-induced killing, enabling Myc to act as a pure growth promoter in this setting.

Studies using animal models support the view that ARF protects cells against Myc-induced tumorigenesis. Mice bearing an Eμ-*Myc* transgene, in which Myc expression is driven by an immunoglobulin heavy chain enhancer, develop Burkitt-type B cell lymphomas with a mean latency of ~30 weeks, and all die of the disease by 1 year of age (99). In the early stages before overt tumors arise, the B-cell compartment of these animals exhibits hyperproliferation, which is balanced by increased apoptosis. When tumors arise, however, the apoptotic index is greatly diminished (100, 101). About 75% of these lymphomas had lesions (*p53* or *ARF* loss, or Mdm2 overexpression) that disabled the ARF-Mdm2-p53 pathway. In addition, when Eμ-*Myc* mice were crossed onto an *ARF*+/− background, tumor progression was greatly accelerated (mean latency, 12 weeks) and 80% of the resulting tumors had lost the wild-type *ARF* allele. Even more strikingly, on an *ARF*-null (100) or *INK4a-ARF*-null (101, 102) background, Eμ-*Myc* transgenic animals all died of highly aggressive lympholeukemias by only 5–6 weeks of age. By contrast, Rb loss of function did not significantly accelerate Eμ-*Myc*-induced disease (101). Most tumors that sustain *ARF* or *p53* mutations do not respond to therapies that can cure mice in earlier stages of lymphoma development (101). Therefore loss of the ability of ARF to modulate the p53 response connotes a poor prognosis, even in those tumors retain wild-type *p53*.

These data imply that by dynamically resetting the effective Mdm2 threshold, ARF reduces the ability of p53 to function in tumor suppression. Consistent with this view, loss of *ARF* makes cells relatively resistant to apoptosis induced by ionizing radiation or cyclophosphamide (94) and can sensitize cells to polyploidy induced by microtubule inhibitors (103). *ARF* loss, like *p53* mutation, can also rescue cells lacking the *Atm* gene from undergoing premature senescence in culture (104), indicating that *ARF* loss modulates the Atm-dependent DNA damage checkpoint. Thus, although DNA damage signals do not appear to activate *ARF per se* (Fig. 4), ARF loss modulates p53 function in such a way as to diminish its accumulation in response to genotoxic stress.

Although the most parsimonious interpretation is that ARF functions in a linear pathway by harnessing the ability of Mdm2 to neutralize p53, there are several reasons to believe that the ARF-Mdm2-p53 pathway has alternative branch points. One line of argument concerns the feedback loops, in which p53 can both induce Mdm2 and repress ARF levels (Fig. 3). The biochemical basis for these connections remains unclear. Moreover, in some *Myc*-induced lymphomas, perturbations were observed that affected expression of more than one gene in the pathway (100). For example, a significant

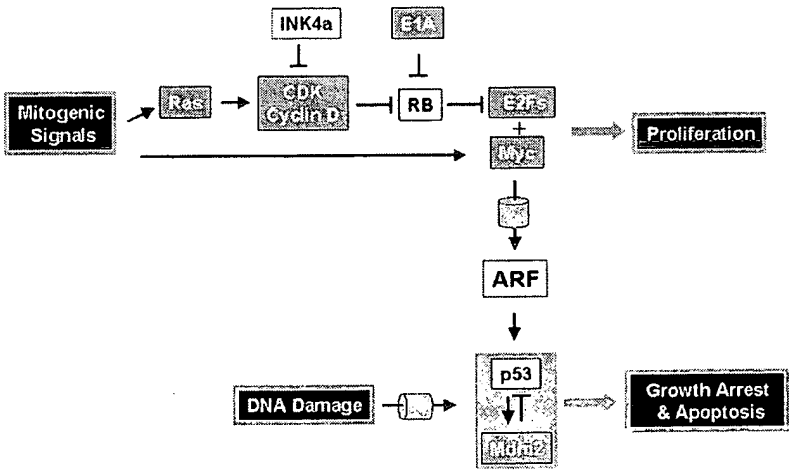

Fig. 4. ARF tumor surveillance. When induced by inappropriate mitogenic signals, ARF antagonizes Mdm2 to activate p53. Hence, hyperproliferative signals are countered by ARF-dependent p53 induction, which diverts incipient cancer cells to undergo growth arrest and/or apoptosis. Loss of the ARF checkpoint (indicated by the *vertical barrel*) subverts this form of cell-autonomous tumor surveillance and allows proteins such as Ras, Myc, E1A, and E2F to function as "pure" proliferation enhancers. DNA damage signals engage various ARF-independent signaling pathways (shown collectively by the *horizontal barrel*) that stabilize p53, most commonly by inducing posttranslational modifications in p53 and/or Mdm2 that prevent their interaction. Although ARF is not directly activated by ionizing radiation or various genotoxic drugs, it is still a potent modifier of the DNA damage response. ARF induction sensitizes cells to DNA damage signals; conversely, ARF loss increases the Mdm2 response and severely dampens the p53 response. All proteins enclosed by *shaded boxes* are potential oncogenes, whereas those illustrated by *unfilled boxes* are tumor suppressors. [This figure is adapted from Sherr (98) and is reprinted with permission from *Genes & Development.*]

fraction of lymphomas exhibited both *ARF* loss and Mdm2 overexpression, implying that both genes can contribute independently to tumor formation. One possibility is that Mdm2 encodes different truncated isoforms, whose as yet undetermined functions may differ from the full-length molecule. At least in principle, ARF might act on targets other than Mdm2, and Mdm2 in turn might regulate proteins other than p53 (Fig. 3). Indeed, there are precedents for the latter, based on reported interactions of Mdm2 with other p53 family members (105), Rb (106, 107), p300 (108), and even E2F1 (109). Much more work is required to critically evaluate these possibilities. Still, it seems evident that disruption of the ARF-Mdm2-p53 pathway occurs frequently in cancers. In humans, *p53* is itself mutated in >50% of cancers, whereas *ARF* loss and Mdm2 overexpression occur in a high fraction of the remaining cases. Hence, disruption of ARF, Mdm2, and p53, like mutations in the $p16^{INK4a}$-cyclin D/cdk4-Rb pathway, again seem to be part of the life history of cancer cells, irrespective of patient age or tumor type.

ARF: In Search of Biochemical Function

ARF is a highly basic protein that localizes to the nucleolus (65, 79, 89). When induced or overexpressed, ARF binds to Mdm2 and imports it into the nucleolus, thereby allowing *p53* to accumulate in the nucleoplasm (89, 110, 111). Recently, ARF was found to bind to a central region of Mdm2 to a segment distinct from Mdm2's nuclear import and export signals, its NH_2-terminal *p53* binding domain, and the COOH-terminal RING domain, the integrity of which is required for E3 ubiquitin ligase activity (111). Both the human and mouse ARF proteins contact Mdm2 through two independent binding sites that are separated by spacer elements of different lengths in the two proteins. In mouse $p19^{ARF}$, the two Mdm2 binding sites cluster in the ARF NH_2 terminus within amino acids 1–37 (111). Segments containing amino acids 1–14 and 26–37 are responsible for cooperative binding and induce an allosteric change in Mdm2 that facilitates its nucleolar import. Interestingly, a cryptic localization signal within the COOH-terminal Mdm2 RING domain contributes to the nuclear import of the ARF-Mdm2 complex. Mutations within this region prevent Mdm2 nucleolar import and instead result in ARF sequestration by Mdm2 in the nucleoplasm (110, 111). The fact that Mdm2 RING domain mutants can oppose the activity of ARF implies that the ARF-Mdm2 interaction is bidirectional, with each protein having a potential to cancel activities of the other.

Although the spacing between the Mdm2 binding domains in the human $p14^{ARF}$ protein is greater than that in mouse $p19^{ARF}$ (88), human or mouse ARF mutants that either do not interact properly with Mdm2 or colocalize Mdm2 to the nucleolus are impaired in arresting cell proliferation (89, 110, 111). To date, these functional data suggest that the ability of ARF to sequester Mdm2 correlates with *p53*-dependent cell cycle arrest. However, it is formally possible that ARF might also antagonize Mdm2 in the nucleoplasm (88). These findings raise interesting questions about the *in vivo* activities of ARF. Is the primary role of ARF to sequester Mdm2 from p53 (89, 110, 111), to interfere with Mdm2-catalyzed ubiquitination (83), to prevent Mdm2 from enforcing p53 nuclear export (87–89, 110, 111), or all of the above?

Conclusions and Future Prospects

In summary, studies over the last decade have indicated that most human cancer cells sustain mutations that affect the functions of Rb and *p53*, either by disabling these genes directly or by targeting genes that act epistatically to prevent their proper function. The *INK4a/ARF* locus surprisingly encodes two products that affect both Rb and *p53*, and the rationale for nature's design of these overlapping tumor suppressors continues to pose a puzzle. An implication may be that the activities of *INK4a* and *ARF* are somehow coregulated through their proximity in the genome, although much of the data collected thus far argue against this interpretation. There is clearly more to learn here.

If, in fact, it is true that disabling the Rb and p53 pathways is a hallmark of cancer, then the most efficacious treatment would be to restore their functions. The inability to specifically target genes to tumor cells and to properly regulate their expression makes "gene therapy" impractical. Novel therapeutics will likely need to target ancillary pathways. Can we take advantage of weaknesses in tumors lacking Rb and/or p53 to selectively kill them? One rationale is based on the concept of "synthetic lethality" in yeast, in which disruption of one gene–in this case, Rb and/or p53–might sensitize cells to disruption of another pathway while sparing cells that retain either one of the two functions. Cyclin A-cdk2 activity is required to terminate E2F function in S phase, and blocking this function triggers apoptosis (112, 113). One idea, then, is that cells that lack Rb and *p53* checkpoints might prove more sensitive than normal cells to cdk inhibitors (114), which are now being widely developed. The recent realization that cells lacking a p53-inducible nuclear subunit of ribonucleotide reductase may rely on a cytoplasmic form of this enzyme to resist drug-induced genotoxic damage (115) may provide another opportunity for targeted therapy. Others have speculated that if specific inhibitors of the cytoplasmic form of ribonucleotide reductase could be developed, these might selectively sensitize cells with mutant p53 to DNA-damaging chemotherapeutic agents (116). Yet another approach would be to activate the apoptotic machinery downstream of the sensory signals that normally lead to *p53*-dependent activation, *e.g.*, by activating death-inducing receptors that couple to caspases (117). An article of faith is that a better understanding of cancer cells will lead to new drug targets and novel therapeutic approaches–that good science will lead to good medicine. Here, again, I quote from Thomas:

"It is much more difficult to be convincing about ignorance concerning disease mechanisms than it is to make claims for full comprehension, especially when the comprehension leads, logically or not, to some sort of action. When it comes to serious illness, the public tends, understandably, to be more skeptical about the skeptics, more willing to believe the true believers. It is medicine's oldest dilemma, not to be settled by candor . . . What it needs is a lot of time and patience, waiting for the science to come in, as it has in the past, with the solid facts" (118).

We should be optimistic that the learning curve is accelerating.

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THE GENETIC BASIS OF HUMAN CANCER

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CONTENTS

CHAPTER 7

Gene Amplification in Human Cancers: Biological and Clinical Significance

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INTRODUCTION

Cytogenetically visible rearrangements in human cancer cells fall into three general categories: (1) deletions, with net loss of genetic material; (2) translocations, with transposition of genetic material but no net loss or gain; and (3) gene amplification, with net gain of a specific chromosomal region. Deletions are thought to represent loss of a suppressor gene, whereas translocations and gene amplification generally represent activation of a proto-oncogene. Translocations could result in inactivation of a suppressor gene, but this appears to be a rare event. Gene amplification also can involve drug resistance genes or other genes that confer a selective advantage when overexpressed. For the purposes of this chapter, gene amplification will be used to refer to an increase in copy number (more than six copies per diploid genome) of a specific, subchromosomal region (generally, 1–2 mb or less). It is not used to refer to numerical gain (generally, three to four copies per diploid genome) of whole chromosomes, chromosome arms, or very large chromosomal regions.

Gene amplification usually is apparent cytogenetically, either as extrachromosomal double-minute chomatin bodies (dmins), or as chromosomally integrated, homogeneously staining regions (HSRs). If the copy number is low (e.g., five to ten copies per cell), if the size of the amplified unit (amplicon) is small, or if the karyotype is extremely complex, gene amplification may not be evident by conventional cytogenetics. However, several different molecular approaches have been developed to allow reliable detection of gene amplification in interphase nuclei from small amounts of tumor DNA (see the following). In general, the latter techniques presuppose that the gene (or genes) that might be amplified are known.

Gene amplification almost always results in the overexpression of one or more genes contained on the amplicon. Usually there is a single gene that appears to be the target of the gene amplification, but some amplified units may contain two or more genes that could theoretically confer a selective advantage. Furthermore, in some cancers, two or more discrete regions may be amplified. However, in the majority of cases, only a single genetic region is amplified in a given tumor.

Theoretically, the amplification and consequent overexpression of a number of genes could confer a selective advantage on a cancer cell. In practice, the majority of examples that have been studied involve oncogenes or drug resistance genes. For the purposes of this chapter, we will concentrate on genes that are amplified in a substantial percentage (at least 10%) of primary tumors that have been extensively studied (at least 50 cases examined). We will discuss *MYCN* amplification in neuroblastomas in detail to illustrate certain points, because it is the most consistent and extensively studied example of oncogene amplification in a human tumor. For the sake of completeness, we will discuss amplification of drug resistance genes in human cancers, but they do not fulfill the criteria for inclusion discussed above.

AMPLIFICATION OF DRUG RESISTANCE GENES

Culturing mammalian cells under conditions of incrementally increasing concentrations of a cytotoxic drug can lead to amplification of the gene encoding the protein that is the target of that drug.[1] This has suggested the possibility that human cancer cells may become resistant to chemotherapy by amplifying certain genes,[2] like the dihydrofolate reductase (*DHFR*) gene in methotrexate resistance, or the multidrug-resistance genes (*MDR1*, *MRP*) in tumors simultaneously resistant to multiple unrelated chemotherapeutic agents.[3] Indeed, there are several reports of human tumor cells that amplified the *DHFR* gene and became resistant to methotrexate.[7,10] However, this has occurred in only a few cases, and only after prolonged clinical treatment, so the frequency with which it occurs does not meet our criteria for inclusion in this chapter. Furthermore, no reports of *MDR* or *MRP* amplification have been reported in human tumors in vivo.[11] Thus, gene amplification appears to be a fairly rare mechanism for the development of drug resistance in humans.

AMPLIFICATION OF ONCOGENES

There are an increasing number of reports of human tumors and cell lines with amplification of proto-oncogenes. A catalog of every reported tumor or cell line that was demonstrated to amplify an oncogene or related genes is beyond the scope of this chapter. Rather, we will focus on examples of gene amplification in pri-

mary tumors that occur with substantial prevalence. Inclusion of cell line studies would bias the data, because established cell lines in many systems show a higher prevalence of gene amplification than their primary tumor counterparts. For instance, amplification of *MYCN* and *ERBB1* (*EGFR*) are found in approximately 80% of cell lines established from neuroblastomas and squamous cell carcinomas, respectively, whereas they are amplified in approximately 25 and 10% of primary tumor specimens, respectively.[12–14]

Many different malignancies have been demonstrated to amplify a variety of oncogenes. These include neuroblastoma, breast, and ovarian carcinoma, small cell lung carcinoma (SCLC), and head and neck squamous cell carcinoma (*HNSCC*). In these malignancies the prevalence of gene amplification ranges from 20 to 50% (see Table 7-1), often with amplification correlating with aggressive behavior or advanced stage. In additional malignancies, such as sarcomas, hepatocellular carcinomas, malignant gliomas, and cervical and gastrointestinal cancers, data are accumulating that implicate gene amplification in their pathogenesis or progression as well (Table 7-1).

Genes amplified in human cancers are thought to confer a growth advantage to a clone, analogous to amplification in vitro of drug resistance genes under certain selective external pressures. Genes frequently amplified in cancer tissues include members of the *MYC* (*MYC, MYCN, MYCL*) and *RAS* (*HRAS, KRAS, NRAS*) families of proto-oncogenes, growth factor receptors (*ERBB*1 and -2, FGFR1 and -2), and genes that are involved in cell-cycle regulation (*CCND*1, *CCNE*, *MDM*2, *CDK*4), in addition to other miscellaneous genes (*AKT*2, *MYB*) (Table 7-2).

Activation of oncogenes is a frequent mechanism of tumorigenesis, and may be accomplished by point mutation, translocation, or amplification. In many malignancies studied to date, amplification of an oncogene is strongly associated with advanced stages of disease and with a poor outcome. In neuroblastoma, for example, *MYCN* amplification is associated with rapid disease progression independent of patient age and stage.[15,17] In other malignancies, however, the presence of amplification does not always maintain significance as an independent variable for outcome when stage and histology are considered. In breast cancer, many studies have correlated the presence of *ERBB*2 amplification with clinical variables such as high stage and grade, lymph node involvement, large tumor size, and steroid hormone receptor absence.[18,19] In multivariate analysis controlling for the preceding clinical data, the presence of *ERBB*2 amplification does not always predict a poor outcome. It remains to be answered, therefore, whether oncogene amplification is a consequence of aggressive, deregulated cell proliferation and resulting genomic instability, or is an early cellular event that is causative of the more aggressive clinical phenotype.

In certain situations, the amplicon harbors multiple candidate genes and it may be difficult to determine a putative oncogene target among these. The most widely studied of these amplicons includes the region of 11q13 containing *CCND1* (Cyclin D1), *FGF*4, *FGF*3/int, *EMS*1 as well as other candidate genes. Investigators have used molecular techniques to implicate *CCND1* based on amplification prevalence and expression patterns in the amplified tumors.[20,21] Other examples include the 12q14 amplicon in the region of *MDM*2, *GLI, SAS, CDK*4; and 8p12 with *FGFR*1, *PLAT*, and others.[22,23] Likewise, it is possible to exploit the presence of amplified domains to discover new oncogenes. Amplified DNA fragments can be cloned and mapped within the genome. From the amplified domain new genes may be sought to explain the biological significance of the amplification, as well as to elucidate their function in normal cells.[20,24]

The prevalence of gene amplification in different tumors, as well as the biological and clinical significance of amplifying particular genes, is discussed in the following, with particular emphasis on the role of *MYCN* amplification in neuroblastoma. Tumors that are presented in detail elsewhere in this volume are discussed only briefly.

MYCN Amplification in Neuroblastomas

Neuroblastomas are tumors of the peripheral nervous system that are found almost exclusively in children. The peak age at diagnosis

Table 7-1 Recurrent Oncogene Amplification in Human Cancers

Tumor Type	Gene Amplified	Frequency, Percent	References
Neuroblastoma	*MYCN*	20–25	(25,26)
Small Cell Lung Cancer	*MYC*	15–20	(57,58)
Glioblastoma	*ERBB*1(EGFR)	33–50	(118,119)
Breast Cancer	*MYC*	20	(18,19,120)
	*ERBB*2(EGFR2)	~20	(18,19)
	*FGFR*1	12	(23,51)
	*FGFR*2	12	(51)
	C*CND*1(Cyclin D1)	15–20	(19,21)
Esophageal Cancer	*MYC*	38	(121)
	C*CND*1(Cyclin D1)	25	(12)
Gastric Cancer	K*RAS*	10	(62)
	C*CNE*(Cyclin E)	15	(21,61)
Hepatocellular Cancer	C*CND*1(Cyclin D1)	13	(122,123)
Sarcoma	*MDM*2	10–30	(16,17)
	*CDK*4	11	(15)
Cervical Cancer	*MYC*	25–50	(125,125)
Ovarian Cancer	*MYC*	20–30	(126,127)
	*ERBB*2(*EGFR*2)	15–30	(53,54)
	AKT2	12	(55)
Head and Neck Cancer	MYC	7–10	(14,15)
	*ERBB*1(EGFR)	10	(14)
	C*CND*1(Cyclin D1)	~50	(14,65)
Colorectal Cancer	*MYB*	15–20	(64)
	H*RAS*	29	(63)
	K*RAS*	22	(63)

Table 7-2 Oncogenes Amplified in Human Cancers

Gene Amplified	Locus	Tumors
AKT2	19q13	Ovarian cancer
CCND1(Cyclin D1)	11q13	HNSCC, esophageal, breast, HCC
CCNE(Cyclin E)	19q12	Gastric cancer
CDK4	12q14	Sarcoma
ERBB1(EGFR)	7p12	Glioblastoma, HNSCC
ERBB2(EGFR2)	17q11	Breast, ovarian cancer
FGFR1	8p12	Breast cancer
FGFR2	10q25	Breast cancer
HRAS	11p15	Colorectal cancer
KRAS	12p13	Colorectal, gastric cancer
MDM2	12q14	Sarcoma
MYB	6q23	Colorectal cancer
MYC	8q22	Ovarian, breast, SCLC, HNSCC, esophageal, cervical cancer
MYCN	2p24	Neuroblastoma

is 22 months, and it is rare after 10 years of age. Neuroblastomas often are localized and have a less aggressive behavior in infants, but they are frequently metastatic and have a poor prognosis in older children. The reason for this apparent discrepancy was unclear until recently. Cytogenetic and molecular analysis of these tumors has identified characteristic differences that allow these tumors to be subclassified into three groups that are distinct in terms of biological features and clinical behavior (see Chapter 22). The feature that characterizes the most aggressive subset of neuroblastomas is amplification of the *MYCN* oncogene.

Cytogenetic examination of neuroblastomas reveals that a substantial number either have extrachromosomal dmins, chromosomally integrated HSRs, or both in subpopulations of cells. These two abnormalities are cytogenetic manifestations of gene amplification. Dmins are the predominant form of amplified DNA in primary tumors, but dmins and HSRs are found with about equal frequency in neuroblastoma cell lines.[25,26] Indeed, dmins and/or HSRs occur in about 90% of neuroblastoma cell lines but only 25% of primary tumors. Evidence suggests that this represents selection *in vitro* for cell lines derived from tumors that have preexisting dmins or HSRs, and there is no evidence to date that these abnormalities develop with time in culture, at least in neuroblastomas. It is unclear why HSRs are a more common form of amplified DNA in established cell lines than they are in primary tumors.

Although cytogenetic analysis of human neuroblastomas frequently has revealed dmins or HSRs in primary tumors and cell lines, the nature of the amplified sequences was not known initially. Evidence for amplification of genes associated with drug resistance was sought, but none was found.[25,26] However, a study was undertaken to determine if a proto-oncogene was amplified in a panel of neuroblastoma cell lines. An oncogene related to the viral oncogene v*MYC*, but distinct from *MYC*, was amplified in the majority of neuroblastoma cell lines tested.[27] The amplified *MYCN* sequence was mapped to the HSRs on different chromosomes in neuroblastoma cell lines, but the normal single-copy locus was mapped to the distal short arm of chromosome 2.[28] Thus, the *MYCN* locus was amplified in neuroblastomas, regardless of whether they had dmins or an HSR, and regardless of the chromosomal location of the HSR.

In collaboration with others, we began a study of primary tumors from untreated patients to determine if *MYCN* amplification occurred. In the initial analysis of 63 primary tumors, amplification ranging from 3 to 300 per haploid genome was found in 24 tumors (38%).[29] All cases with *MYCN* amplification in this initial study came from patients with advanced stages of disease. The progression-free survival of these patients was analyzed according to the stage of disease and *MYCN* copy number.[30] *MYCN* amplification clearly was associated with rapid tumor progression and a poor outcome, independent of the stage of the tumor.

These studies have been extended to over 3000 patients with neuroblastoma enrolled in protocols of the Children's Cancer Group (CCG) and the Pediatric Oncology Group (POG) (Table 7-3).[26,31] Examples of *MYCN* amplification seen in some of the primary tumors are shown by fluorescence *in situ* hybridization (FISH) (Figs. 7-1B, 7-1C), compared to a normal control (Fig. 7-1A). It is now clear that, among patients with less advanced stages of disease traditionally associated with a good prognosis, a minority (5–10%) have tumors with *MYCN* amplification.[26,31] Our data indicate that virtually all of these patients are destined to have rapid tumor progression and a poor outcome, similar to their counterparts with advanced stages of disease. Over 30% of patients with more advanced tumor stages had *MYCN* amplification, and they also had an expectedly poor outcome. Our findings that *MYCN* amplification is associated with a poor outcome regardless of the clinical stage of tumor is supported by independent studies from Japan and Europe.[32–34]

We analyzed the *MYCN* copy number in multiple simultaneous or consecutive samples of neuroblastoma tissue from 60 patients to determine whether or not the presence or absence of *MYCN* was consistent in different tumor samples from a given patient, or if single-copy tumors ever developed amplification at the time of recurrence.[35] Indeed, we found a consistent pattern of *MYCN* copy number (either amplified or unamplified) in different tumor samples taken from an individual patient, either simultaneously or consecutively.[35] These results suggest that *MYCN* amplification is an intrinsic biological property of a subset of neuroblastomas. Tumors that develop *MYCN* amplification generally do so by the time of diagnosis, and so cases of neuroblastoma with a single copy (per haploid genome) of *MYCN* at the time of diagnosis almost never have developed amplification subsequently.

About 25–30% of the children with neuroblastoma have *MYCN* amplification in their tumors, and virtually all of these children have rapidly progressive and fatal disease. However, in patients with single-copy tumors, there is not yet a biological marker or explanation why half of these patients do not survive. A general correlation has been demonstrated between *MYCN* copy number and expression, but a high level of *MYCN* expression in single-copy tumors does not appear to identify a subset with a particularly poor outcome.[34,36–38] It is still possible that activation of *MYCN* by mechanisms other than amplification or overexpression may play an important role. In addition, it is likely that either activation of other oncogenes, deletion of specific suppressor genes, or other genetic lesions may contribute to the poor clinical outcome in these patients.

We had sought evidence for amplification of other oncogenes, including *MYC, MYCL, NRAS, HRAS, KRAS, EGFR1, EGFR2, SIS, SRC, MYB, FOS*, and *ETS* in neuroblastomas, but none was found.[26] However, there are at least six examples of neuroblastoma cell lines or primary tumors that amplify regions that are remote from the *MYCN* locus at 2p24. These include amplification of

Table 7-3 Correlation of *MYCN* Copy Number with Stage and Survival in 3000 Neuroblastomas

Stage at Diagnosis	*MYCN* Amplification		Three Year Survival, Percent
Benign Ganglioneuromas	0/64	(0%)	100
Low Stages (1,2)	31/772	(4%)	90
Stage 4-S	15/190	(8%)	80
Advanced Stages (3,4)	612/1,974	(31%)	30
TOTAL	658/3000	(22%)	50

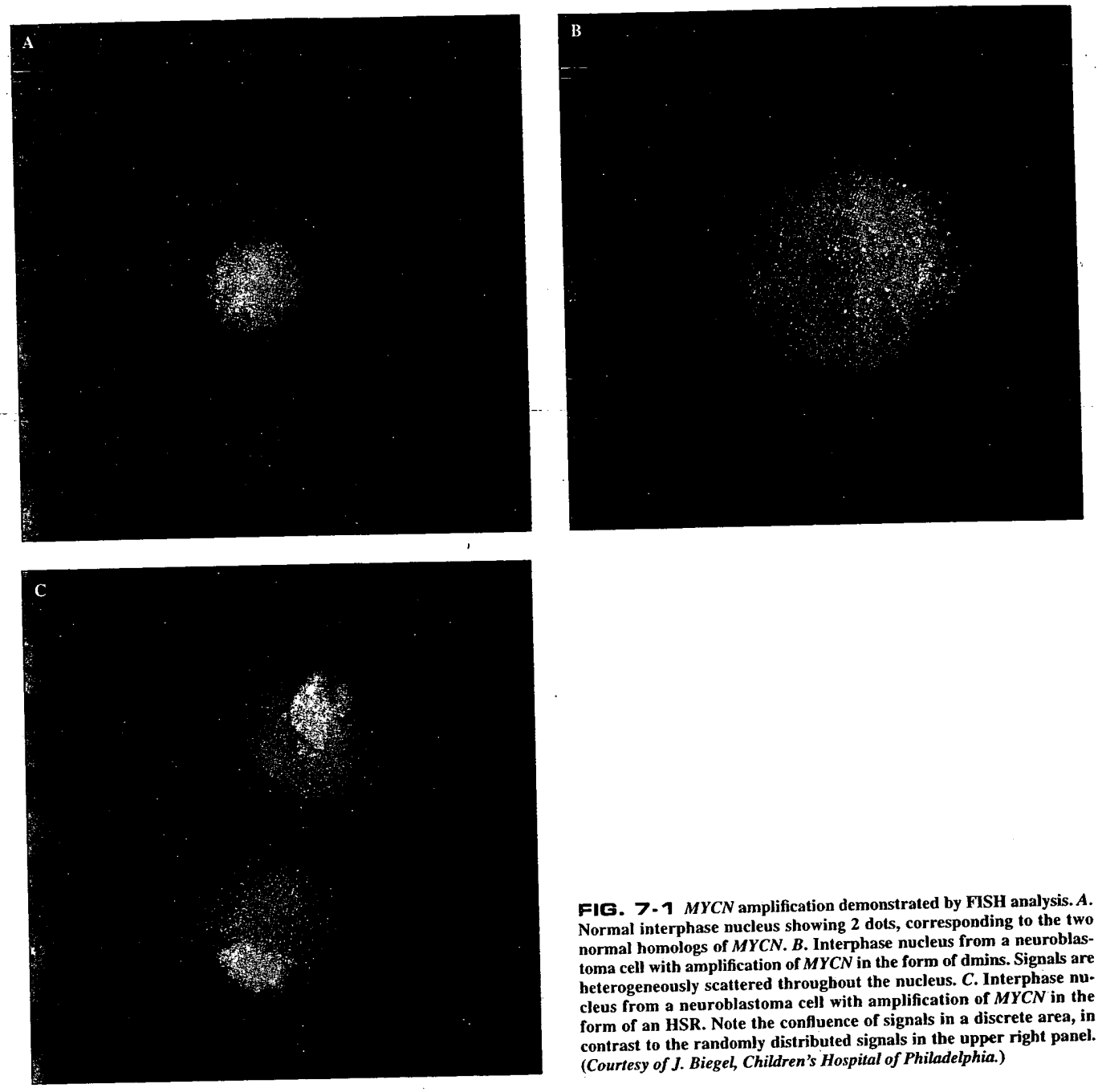

FIG. 7-1 *MYCN* amplification demonstrated by FISH analysis. *A.* Normal interphase nucleus showing 2 dots, corresponding to the two normal homologs of *MYCN*. *B.* Interphase nucleus from a neuroblastoma cell with amplification of *MYCN* in the form of dmins. Signals are heterogeneously scattered throughout the nucleus. *C.* Interphase nucleus from a neuroblastoma cell with amplification of *MYCN* in the form of an HSR. Note the confluence of signals in a discrete area, in contrast to the randomly distributed signals in the upper right panel. *(Courtesy of J. Biegel, Children's Hospital of Philadelphia.)*

DNA from 2p22 and 2p13 in the IMR cell line, as well as coamplification of both *MYCN* (from 2p24–32) and *MDM2* (from 12q14) in the NGP, TR, and LS cell lines.[22,39–41] Finally, there is one report of coamplification of *MYCN* and *MYCL* in a neuroblastoma cell line,[42] and this has been seen in at least one primary tumor as well (unpublished observations). These findings indicate that more than one locus can be amplified, but no neuroblastoma has been shown to amplify another gene that did not also amplify *MYCN*.

The presence of *MYCN* amplification in human neuroblastomas has been shown to correlate strongly with advanced clinical stage and poor prognosis.[26,29,30,33,34,36–38] Our recent studies showed a very strong correlation between *MYCN* amplification and 1p LOH ($p < .001$), indicating that LOH was common in patients with amplification.[25,26,43] Both *MYCN* amplification and deletion of 1p (as detected by cytogenetic or molecular analysis) appear strongly correlated with a poor clinical outcome and with each other, but it is not yet clear if they are independent prognostic variables.[44–50] Nevertheless, they appear to characterize a genetically distinct subset of very aggressive neuroblastomas.

ONCOGENE AMPLIFICATION IN OTHER CANCERS

Breast Cancer

Breast cancer is the single most common cancer occurring in women, and represents a genetically heterogeneous disease with frequent amplification of oncogenes and allelic deletions.[18] *MYC*, *ERBB2*, and *CCND1* (Cyclin D1) are the most frequently amplified genes in breast cancer, each occurring in approximately 20% of cases. Genes of the fibroblast growth factor receptor family (*FGFR1* and *FGFR2*) are also amplified in another 12% of cases,

respectively, although it is not clear that their expression is increased above baseline (Table 7-2).[23,51] Many studies have attempted to correlate the presence of a particular amplified gene with outcome or other clinical prognostic factors with contradictory results, although there is support for the notion that amplification is a late event in the multi-step pathogenesis of breast cancer.[18,52]

Ovarian Cancer

In ovarian cancer, amplification of *ERBB2* and *MYC* are each found to occur in 15–30% of samples.[53,54] *ERBB2* or *MYC* amplification tends to occur in advanced stages of disease and is seen infrequently in early invasive or borderline ovarian epithelial tumors, again implying that these are late genetic events.[53] *AKT2* is a protein serine/threonine kinase gene discovered by homology to *VAKT*, a viral oncogene that can cause lymphomas in mice. This oncogene is amplified independently of *MYC* or *ERBB2* in approximately 12% of ovarian cancers and also correlates with invasiveness.[55] Although amplification of *NRAS* and *KRAS* are seen infrequently in ovarian cancers, there is some evidence that these events occur earlier based on their prevalence in low stage disease, but this remains speculative.[56]

Lung Cancer

Lung cancer is one of the most common and fatal malignancies, and its incidence is increasing in both men and women. Small cell lung carcinoma (SCLC) makes up approximately 25% of cases and has a distinct clinical course with a propensity for early metastasis. The *MYC* proto-oncogenes are the only genes amplified in a significant number of SCLCs (15–20% of cases), although cell lines derived from *SCLC* specimens more frequently have gene amplification.[14,57,58] The majority of these cases involves *MYC*, with amplification of *MYCN* and *MYCL* also occurring.[57–59] However, coamplification of multiple *MYC* family genes has not been described to date in *SCLC* specimens. It is possible that the propensity of *SCLC* cell lines to amplify *MYC* in vitro illustrates this cancer's underlying genetic instability and virulent phenotype. The clinical association of *MYC* amplification with shortened survival was seen in one study, but this has not been confirmed.[60]

Gastrointestinal Cancers

A diverse group of genetic alterations occur in gastrointestinal cancers, involving mutation or deletion of tumor suppressor genes and DNA repair genes, in addition to amplification of oncogenes. Amplification of *MYC* and *CCND1* occurs frequently in esophageal squamous cell carcinoma, the latter gene being almost uniformly amplified in metastatic disease and correlating with clinical and pathological staging.[61] In contrast, a plethora of genetic alterations occur in gastric cancer, many presumed to induce cell proliferation via induction of growth factor production or increased growth factor receptor expression, but amplification does not occur commonly. These genetic alterations differ in poorly-differentiated and well-differentiated gastric cancers, and overall 10–15% of cases will demonstrate *KRAS* or *CCNE* amplification.[21,61,62] In colorectal cancers, cytogenetic evidence for gene amplification has long existed in the form of dmins but with no clear candidate genes. In some series, *MYB*, *HRAS*, or *KRAS* amplification has been found, but in the majority of colorectal cancers, none of the known oncogenes have been amplified in a significant proportion of cases.[63,64]

HNSCC, Other

In head and neck squamous cell carcinomas, amplification of the 11q13 locus with *CCND1* occurs frequently, whereas *MYC* and *ERBB1* amplification are also seen.[14,65] These findings are particularly germane in that current clinical prognostic factors are poor for *HNSCC* and molecular data may in the future become of significant predictive importance. In still other malignancies, gene amplification has been shown to be a prominent feature. In glioblastoma, hepatocellular carcinoma, cervical cancer, and certain sarcomas the role of oncogene amplification is being elucidated as advanced molecular analyses of these malignancies becomes more prevalent (Table 7-2).

MECHANISMS OF GENE AMPLIFICATION

The precise mechanism by which gene amplification occurs in human cancers is not known with certainty. Some information can be obtained by studying amplification of drug resistance genes in cells grown in vitro under selective pressure. However, these systems generally involve the rapid selection for resistance to an antimetabolite or other toxic compound, so it is not clear that these model systems will provide relevant information. It is more likely that the selective pressures that lead to amplification of a gene that provides a survival or growth advantage in vivo are not as profound, so the mechanisms leading to gene amplification may be quite different.

Unfortunately, only the end point of gene amplification can be studied, so it is difficult to draw firm conclusions about early events in the amplification process. Some information can be obtained by structural analysis of the amplified unit, as well as analysis of the genomic configuration of the locus that was amplified. In the majority of cases, the initial form of amplified DNA seen in human cancers is the extrachromosomal dmin. These structures lack centromeres or kinetocores, and they apparently segregate randomly in the two daughter cells after cell division. In order to remain stable, it is likely that dmin are closed circular molecules. Experimental data supporting this hypothesis have come from the study of amplified units involving the *MYCN* gene in human neuroblastomas, as well as other systems.

Several models have been proposed to explain the genomic amplification of specific chromosomal regions. These include the overreplication (onion skin) model, the chromosomal breakage/deletion plus episome formation model, and the duplication and crossing over model. These models were based on the analysis of genomic rearrangements that followed the rapid, stepwise selection of resistance to a particular drug. However, none of these models are consistent with what is known about the structure of DNA in tumors with spontaneous amplification of regions containing oncogenes, such as the *MYCN* oncogene in human neuroblastomas.

Amler and colleagues used pulsed-field gel electrophoresis (PFGE) to study the restriction pattern of *MYCN* amplicons using infrequently cutting enzymes and probes around the *MYCN* gene.[66,67] These studies led to the conclusion that the amplicons were arranged in tandem, head-to-tail arrays in the HSRs, and that most of the amplicons had a consistent restriction pattern (there was relatively little rearrangement). These conclusions were verified by Schneider and colleagues who cloned a representative amplified domain into yeast artificial chromosomes (YACs) from a cell line with 300 copies of *MYCN* per cell as dmins.[68] They showed that the amplicons were arranged in a head-to-tail, presumably circular arrangement, with general preservation of the germ line genomic structure. Finally, Corvi and coworkers showed the germ line copy of

MYCN was apparently intact on both homologs of chromosome 2 (i.e., it was not deleted).[69]

Based on the overreplication model, the resolution of this complex structure would result in considerable rearrangement of the amplified sequences relative to the germ line configuration. Also, a gradient of amplification should be seen, with the highest level of amplification near the gene that is the presumed target of amplification, with decreasing amplification further away from the amplification epicenter. However, the PFGE data by Amler and YAC cloning data by Schneider are inconsistent with this model, even though some variation from the germ line pattern was detected by both approaches.[66,68]

The duplication plus crossing over model would suggest that relatively intact copies of the amplicon would occur *in situ* on a given chromosome, leading to an HSR at the site of the normal gene. However, the most common form of amplified DNA is the extrachromosomal dmins, and the chromosomal locations of HSRs representing amplification of *MYCN* in neuroblastomas occur almost everywhere but at the normal chromosomal location of *MYCN* at 2p24.[70] Thus, this model does not seem applicable to what happens when *MYCN* becomes amplified in human neuroblastomas, and perhaps amplification of oncogenes in general.

Finally, the chromosomal breakage/deletion plus episome formation model would suggest that one germ line copy of the amplified region is deleted, but this was not detected in the five cell lines studied by Corvi or the cell line studied by Kanda and Shiloh.[39,69,71,72] One study by Hunt and Tereba did suggest that one germ line copy of *MYCN* was deleted from a homolog in one cell line by segregating the homologs into separate somatic cell hybrids, but this is inconsistent with data from at least six other cell lines studied by FISH.[73] It is possible that the apparent deletion may have occurred during the formation of the somatic cell hybrids, or that there may be different mechanisms by which *MYCN* amplification can occur.

In summary, none of the above models derived from the study of amplification of drug resistance genes appears to apply to the *de novo* amplification of oncogenes in human tumors in vivo. It seems likely that a variation of the breakage/deletion plus episome formation model is applicable. However, given the apparent retention of the normal parental copies of the amplified region, it is likely that some form of duplication of the amplified region occurs, followed by excision and circularization to form a dmin.[73a] Further study will be required to elucidate this mechanism, which may be difficult if a model system for *de novo* oncogene amplification cannot be found.

STRUCTURAL ANALYSIS OF AMPLICONS

General Comments about the Size/Structure of Amplified Domains

Estimates of the size of the amplified domain around the *MYCN* proto-oncogene in neuroblastomas have ranged from 300–3000 kb, based on physical, chemical and electrophoretic measurements of the amplified DNA.[70] However, all these approaches to map the size of the amplified domain have been indirect. An attempt was made to clone and map the amplified domain around *MYCN* in a representative neuroblastoma cell line NGP using cosmid and lambda vectors.[74,75] However, only 140 kb of contiguous DNA around the *MYCN* locus could be mapped, and a number of additional amplified clones were identified that were not contained in the 140 kb contiguous region. The entire 140-kb contiguous locus was amplified in a panel of 12 primary neuroblastomas with *MYCN* amplification, whereas the noncontiguous fragments were amplified in subsets of them.[75] These data indicate that, although each tumor had a relatively unique pattern of amplified DNA fragments, there was core region that was consistently amplified in different tumors.

Amler and Schwab have analyzed the amplified domain of a series of neuroblastoma cell lines with *MYCN* amplification, most in the form of chromosomally integrated HSRs.[66] They analyzed the amplification domain by pulsed-field gel electrophoresis and hybridization with DNA probes that represent the 5′ and 3′ ends of the *MYCN* gene. They confirmed the heterogeneity of size of the amplified domain seen in different neuroblastomas demonstrated by earlier studies.[39,72,74,75] They also concluded that most amplified regions of DNA consisted of multiple tandem arrays of DNA segments that were several hundred kilobases in size, and that *MYCN* was at or near the center of the amplified units. Rearrangements were more commonly found in the cell lines with higher *MYCN* copy number (greater than 50–100 copies/haploid genome).

We have analyzed the amplified domain in human neuroblastomas in order to determine the size and structural organization of this region in different tumors and cell lines, as well as the core region that is consistently amplified. Because of the large size of the domain, we have used the yeast artificial chromosome (YAC) cloning vector system.[76] To date, 20 YACs have been identified which contain segments of the amplified domain from a representative neuroblastoma cell line, and the YACs can be arranged in a contiguous linear map of approximately 1.2 Mb.[68,77] In general, the YAC clones are consistent with a linear map of the region, but a few rearrangements have been identified thus far. Our data also indicate that the core of the domain amplified in different tumors is no more than 130 kb, and the amplicons of one tumor have deleted about half of this core.[78] Although it remains a formal possibility that there may be other genes near *MYCN* whose expression is important in mediating the aggressive phenotype associated with *MYCN* amplification, our data suggest that *MYCN* is the target of amplification in neuroblastomas.

Combined with the findings of Corvi and others that both homologs of *MYCN* are retained, these data are inconsistent with the deletion model of gene amplification.[79–81] Nevertheless, it is likely that a large region containing the selectable marker and an origin of replication is duplicated, excised, and circularized, forming an extrachromosomal episome (Fig. 7-2). This episome segregates randomly during cell division, but cells with more copies of the marker gene have a selective advantage and accumulate to a certain stable average number. Although there would inevitably be heterogeneity in the number of episomes per cell, the average number in a population should be relatively stable. Larger episomes or episome multimers would be visible with the light microscope and called dmins. As a rare event, the episomes would integrate into a chromosome at an apparently random site forming an HSR. Because of the secondary recombinational events, the structure of the amplified domains from HSRs would likely be more rearranged and heterogeneous.[68,77]

BIOLOGICAL SIGNIFICANCE OF GENE AMPLIFICATION

Presumably, the mechanism by which gene amplification confers a selective advantage on the cancer cells is that there is overexpression of the gene or genes contained on the amplicon. In general, this overexpression is proportional to the increase in copy number, but this is not an absolute correlation. The overexpression of the gene or genes then must confer some advantage to the cell in terms

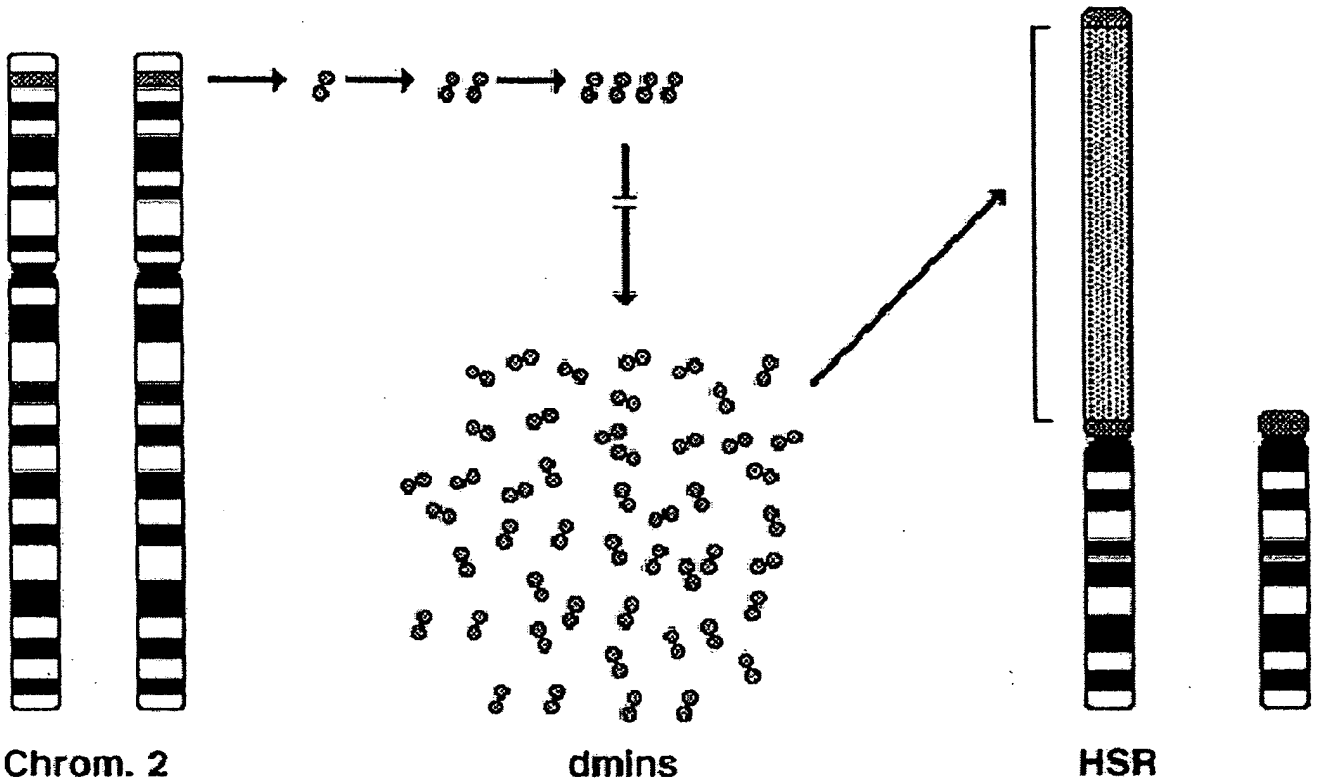

FIG. 7-2 Hypothetical process of *MYCN* amplification in human neuroblastomas. The location of the normal *MYCN* gene is shown by the shaded band, chromosome 2p24 on the left. The current data suggest that a large region of DNA containing the *MYCN* gene is duplicated on one homolog of chromosome 2 and forms an extrachromosomal element or episome, which probably is circular. It must contain an origin of replication but lacks a centromere or kinetocore, so it does not segregate evenly between daughter cells during mitosis. However, since it seems to provide a growth advantage in vivo and in vitro, the episomes accumulate in a subset of cells but remain unstable and therefore heterogeneously distributed among the cells in the population. If these episomes are large enough to be seen in the light microscope they are called dmins. As a rare event, particularly in cells in vitro with pre-existing *MYCN* amplification, the dmins linearly integrate into a chromosome at a seemingly random site.

of growth or survival. We will review briefly what is known about the likely consequences of overexpressing the most frequently amplified genes in human cancers: the *MYC* family, the *RAS* family, the *ERBB* and *FGFR* families, and the cell-cycle control genes, including the cyclins and *MDM2*.

MYC Family Amplification

The structure of the *Myc* proteins consists of a transactivating domain at the N-terminal third of the protein, followed by a basic region, helix-loop-helix region, and leucine zipper domain (B-HLH-Zip). These proteins are thought to activate transcription by binding to a hexanucleotide motif CACGTG known as an E-box. However, they do not bind well as monomers or as homodimers, but rather as heterodimers with another B-HLH-Zip protein known as *MAX*.[82] This protein lacks a transcriptional activation domain, and it can form homodimers that are thought to be transcriptionally repressive.[83–87] Thus, in a state of *MAX* excess, *MAX* homodimers predominate, and transcription is repressed. Conversely, when *N-MYC* (or C-*MYC*) are expressed at higher levels, heterodimers form, resulting in transcriptional activation. The consequence of this is progression through the cell cycle, and proliferation of the cell population. Inactivation of *MYCN* occurs when leucines in the Zip region are mutated, indicating the importance of this region in *MYCN* function.[88]

MYC oncoproteins have a short half-life (20–30 minutes), so once transcription and translation cease, the levels of *MYC* fall rapidly, and *MAX-MAX* transcriptional repression predominates. However, in tumors which amplify *MYC, MYCN* or *MYCL*, the level of amplification is usually one to two orders of magnitude (or more), with corresponding overexpression of the oncoprotein.[34,36–38,58] This leads to very high steady-state levels of MYC, even when it is not being actively transcribed. This in turn presumably favors a state of proliferation, with less likelihood that the cell will enter G_0 and become quiescent. This is presumably the selective advantage conferred by overexpressing this family of genes.

RAS Family Amplification

RAS genes encode a family of proteins known as G proteins, which participate in the signaling cascade initiated by the activation of tyrosine kinase receptors or other mechanisms.[89–91] *RAS* activation (by certain base pair mutations) or overexpression (usually by amplification) leads to enhanced signal transduction through the *RAF*1 serine-threonine kinase, the early response kinases (*ERK*1 and *ERK*2), and subsequent induction of transcription of immediate-early genes (e.g., *FOS, JUN, EGR*1, etc.) in the nucleus.[89–91]

The amplification and overexpression of *RAS* genes leads to constitutive signal transduction, mimicking the effects of continual activation of a growth factor receptor, like *EGFR* or *PDGFR*.[89–94] This in turn leads to continuous cell proliferation. However, the cellular background is very important, because in certain cellular milieus (such as neural cells), the predominant receptor tyrosine kinase may be signaling differentiation and not proliferation.[95–97] Activation or overexpression of *RAS* in this context would lead to differentiation of the cell, which would not promote the proliferation of tumor cells.[89–91,93] This may explain why *RAS* activation (usually by mutation) is rare in neural tumors, whereas it is one of

the most common types of oncogene activation in many other tumor types.[92-94]

Amplification of the ERBB and FGFR Family

Amplification and overexpression of genes for growth factors and/or their receptors, such as those of the *EGF/EGF*R (*ERBB*) and *FGF/FGFR* families, occur in a number of human cancers.[23,51,53] The *ERBB* and *FGF* receptors are transmembrane protein tyrosine kinases involved in cell proliferation. After specific ligand binding, signal transduction occurs through phosphorylation of the SH2 domains of cytoplasmic proteins associated with the receptor. This leads to *RAS* activation, serine/threonine phosphorylations, and changes in phosphotidyl inositol metabolism with the end result being modulation of specific genes necessary for proliferation.[98,99]

Gastric cancers express a number of growth factors and receptors including *EGF, TGF-α, ERBB2*, and *FGFR2*.[61,100] *EGF* is synthesized as a transmembrane precursor with secreted protein being released by proteolytic cleavage. It has been shown to enhance growth of cells from most epithelial tumors, and in gastric cancer *EGF* amplification is associated with poor outcome.[100,101] In addition, human gastric cancers that possess both *EGF* and *EGF*R or *ERBB2* simultaneously had a greater degree of local invasion and lymph node metastasis, further suggesting autocrine stimulation. Additionally, high levels of expression of growth factor receptor alone may result in autophosphorylation and signaling even in the absence of ligand.

Constitutive activation of these growth factor-receptor signaling pathways is a common motif in oncogenesis.[98,99] Amplification and overexpression of either ligand or receptor may cause growth stimulation in an autocrine or paracrine fashion in the appropriate cellular setting and contribute to biologic malignancy.

Amplification of the Genes Encoding Regulators of the Cell Cycle

Cells of most higher organisms maintain a stringent checkpoint control over progression from G1 into S phase and subsequent cell division. Early in G1, cells are dependent on mitogenic stimuli, but beyond a certain point a switch to intrinsic cell-cycle machinery occurs with a reduced requirement for growth factors, apparently ensuring an ordered completion of the cell division cycle. This switch-point is mediated by the D-type cyclins, though many proteins play important roles as both positive and negative regulators. These include (but are not limited to) other cyclins, cyclin-dependent kinases (CDKs), and their inhibitors (CDKIs). Activation of G1 cyclins (*CCND1* and *CCNE*) occurs via growth factor signals which induce *CCND1* phosphorylation. Activated cyclins D and E, in association with their predominant cyclin-dependent kinases CDK4 and CDK2, then sequentially phosphorylate the RB protein. This causes the release of E2F from pRB, which activates transcription of genes involved in cell proliferation.[102,103] Overexpression of *CCND1, CCNE*, or *CDK4* presumably results in a growth advantage for cells by tipping the balance in favor of G1 transition rather than quiescence. Likewise, overexpression of *MDM2* could have similar effects. *MDM2* protein binds p53, a potent cell-cycle inhibitor. By blocking p53 transcriptional activation of CDKIs such as p21, cells are more likely to enter S phase.[102,103] This loss of checkpoint control fails to allow time for repair of DNA damage caused by a multitude of insults, such as ionizing radiation, drugs, and cellular toxins. Experimentally, cells with either homozygous loss or inactivation of p53 or *CCND1* overexpression have been shown to more readily undergo gene amplification in response to selective pressures.[104-106] This enhancement of genetic instability illustrates the potential importance of cell cycle-control protein perturbations in the development of the malignant phenotype.

METHODS OF DETECTING GENE AMPLIFICATION

A variety of techniques may be used to detect gene amplification.[107] Each has certain advantages and disadvantages in terms of the amount of tumor tissue or DNA needed, the ease of performing the technique, the sensitivity of the technique to detect low levels of amplification, or the size of the amplified unit. These techniques include: conventional cytogenetics; Southern analysis; fluorescence in situ hybridization (FISH); semiquantitative PCR; and comparative genomic hybridization (CGH).

Cytogenetic Analysis

Cytogenetic analysis is a labor-intensive technique that is dependent on dividing cells in the tumor tissue or adaptation to growth in short-term culture. As a result, it is unsuccessful in the majority of solid tumors and a substantial minority of leukemias. The detection of HSRs or dmins provides evidence for gene amplification in the culture, although small HSRs or dmins may escape detection. Also, it is impossible to know with certainty which gene or chromosomal region is amplified. This is a useful technique when investigating a new tumor type but is not the most efficient or sensitive approach once it is known which gene or genes are likely to be amplified.

Southern Analysis

Southern analysis is perhaps the gold standard by which other techniques are compared. This technique relies on the preparation of DNA from tumor tissue that is relatively free of contaminating normal tissue. The DNA is digested with a restriction enzyme, electrophoresed on an agarose gel, blotted to a membrane, and hybridized with a radioactive probe corresponding to the gene or genetic region thought to be amplified.[29,35] This technique is rather labor intensive, and it requires at least 5–10 μg of DNA. Frequently an internal control gene is also hybridized so the intensity of the band of interest can be normalized for quantitative densitometry. However, this technique can miss a small percentage of amplified cells in an unamplified population. Slot-blot is a variation on this technique that requires no digestion, only 1 μg of DNA, and it is easily subjected to densitometric analysis, but low levels of amplification can be missed.

Fluorescence In Situ Hybridization (FISH)

The FISH technique is probably the most efficient and popular technique at the current time.[108,109] It requires only a small amount of tumor tissue, usually a touch prep or cytospin of several thousand cells on a slide. It can even be done on paraffin embedded tissue.[109] Hybridization to interphase nuclei takes place overnight under a coverslip, and the results can be interpreted within 48 hours. This technique can also distinguish a small percentage of amplified tumor cells in a population of normal or nonamplified tumor cells, if a counterstain to visualize the cells is implemented. It may be necessary to utilize a control probe for the centromere or opposite

chromosomal arm of the region usually amplified in order to distinguish between low-level amplification and polysomy for the particular chromosome. However, this approach does require an expensive fluorescence microscope and sophisticated imaging equipment, and the probes are expensive to purchase commercially, so it is not the ideal approach for all laboratories.

An interesting variation of this technique has been developed whereby amplicons are microdissected and used as FISH hybridization probes to determine the chromosomal origin.[110] This micro-dissection approach allows the chromosomal origin of dmins or HSRs to be determined in a single hybridization without knowing a priori the genetic region that is amplified. Indeed, this approach can also identify amplification of previously unsuspected chromosomal regions. However, in addition to the technical demands of FISH, this approach also requires both successful metaphase preparation, with identification of dmins or HSRs, and the ability to perform microdissection and preparation of a microclone library. Therefore, this method will not be useful for most laboratories.

Semiquantitative PCR

The PCR technique had obvious advantages that might be applied to the detection of genomic amplification in small amounts of tumor DNA.[111-114] As long as the number of cycles of amplification is carefully controlled, and an internal control gene is used for normalization, it is possible to semiquantitatively amplify a given gene or DNA sequence and distinguish the normal copy number from multiple copies. Although some claim to detect as low as two-fold amplification, generally five- to ten-fold amplification is the limit of detection of this semiquantitative technique on primary tumor samples.[111-114]

Comparative Genomic Hybridization (CGH)

This is the newest of the approaches that has been applied to the detection of genomic amplification.[115-117] This approach has the advantage of conventional cytogenetics, in that the whole genome is surveyed, not just one or a few specific genomic regions that are known or suspected to be amplified in a given tumor type. Also, because the chromosomal location of the amplified region is known, the likely gene amplification frequently is apparent. Tumor metaphases are not needed, and only a small amount of DNA is required. However, this approach requires a sophisticated fluorescence microscope and image capturing capability, as well as software to analyze the data obtained. Furthermore, very small amplicons or low levels of amplification may be missed.

SUMMARY AND CONCLUSIONS

Gene amplification in human cells is a phenomenon that appears to be restricted to tumor cells. In the majority of cases in which the amplified genomic region has been identified, the driving force of the amplification appears to be an oncogene, usually of the *ERBB, MYC*, or *RAS* families. A variety of other genes have been shown to be amplified in small numbers of cases, or in tumor cell lines, but the above families are found the most consistently. Furthermore, examples of amplification of genes conferring drug resistance have been found in certain cancers at relapse, but this does not appear to be a common mechanism by which cancer cells become drug resistant in vivo.

The mechanism by which amplification of oncogenes in human cancer cells occurs is unknown, but it probably involves the duplication of a large chromosomal region, followed by deletion and circularization to form an extrachromosomal dmin. Then there is accumulation of these dmin by uneven segregation into the daughter cells during mitosis, until maximal advantage is achieved. This is presumably a consequence of the overexpression of a gene or genes on the amplicon that confer the selective advantage. The region amplified may be quite large, from 100 kb to several Mb. However, the region that is consistently amplified may contain little more than the single gene suspected of providing a growth or survival advantage.

The identification of oncogene amplification in certain human cancers provides some insight into the pathogenesis of these diseases. Indeed, in some tumor systems, oncogene amplification had been associated with a greater likelihood of invasion, metastasis and a poor outcome. Thus, the identification of oncogene amplification in human cancers may have some prognostic value. Ultimately, it may be possible to develop novel therapeutic approaches that target the amplified oncogene or the overexpressed oncoprotein. This approach may be particularly attractive if the amplified gene is mutated or chimeric, allowing the development of selective biological reagents, including antibodies, drugs, antisense, or targeted gene therapy approaches.

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